Removal of Flatulence Causing Sugars in Soymilk

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ABSTRACT

The enzyme, α-galactosidase was extracted from peanuts using various buffers and solvents. The specific activity of soluble preparation of peanut ranged from 0.0083 to 0.0606 U/mg. Phosphate buffer extract showed the maximum specific activity of 0.0606 U/mg. The soluble α-galactosidase was immobilised in calcium alginate beads and the specific activity of the immobilized enzyme was found to be 0.0808 U/mg. Activity yield was 33%. The immobilised enzyme showed increase in activity compared with the soluble enzyme above 35°C. The thermal stability of the immobilised α-galactosidase was significantly improved in comparison to the soluble form. The effect of different pH depicts that at acidic pH the activity of the immobilised α-galactosidase was higher than that of the soluble enzyme. The K_m and V_{max} for immobilised enzyme was higher when compared with the soluble enzyme. Immobilised α-galactosidase retained 20 % activity after 7 repeated uses. The immobilised enzyme exhibited high storage stability. The immobilised enzyme was used in batch and continuous packed bed reactors for the hydrolysis of stachyose and raffinose in soymilk. Flatulence causing raffinose and stachyose, after hydrolysis, was removed to a remarkable extent. Hydrolysis of flatulence causing oligosaccharides of soymilk in a continuous packed bed reactor has not been attempted previously.

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1. INTRODUCTION

Soymilk has proven to be an excellent alternative for bovine milk among lactose intolerant people all over the world. Ingestion of a large dose of lactose through consumption of milk products causes diarrhoea, bloating, and flatulence in humans depending on the severity of their lactose intolerance. Soymilk is rich in protein and has a well-balanced amino acid pattern. Despite having many advantages, soymilk is not widely accepted because of its flatulence inducing effect upon sensitive individuals. This effect is due to the presence of non-digestible galactosaccharides such as stachyose and raffinose.

Humans lack the enzyme α -galactosidase, which is required to hydrolyse α -1, 6 bonds of raffinose and stachyose. The undigested raffinose and stachyose enter the colon and can cause intestinal flatulence when anaerobic microorganisms ferment these galactosaccharides producing carbon dioxide, hydrogen and methane. This can result in discomfort, headaches, dizziness, and slight mental confusion, reduced ability to concentrate, slight retinal edema, diarrhoea, dyspepsia, constipation and painful contraction of the colon.

Various attempts have been made to reduce the flatulence factors. The use of α-galactosidase is one of the methods. Although α-galactosidases have been extracted from plants, animals and microorganisms, only a few are available commercially, which are very expensive. Peanuts have shown to be a cheap and potential source of α-galactosidase.

The application of active enzymes is not always possible as purified enzymes are costly and have to be discarded after each use, which is not economical. The immobilisation of enzymes offers several advantages over the

use of soluble enzyme mainly due to their reusability and thereby reducing the production cost. They can be used as selective absorbents for purification of proteins and enzymes, fundamental tools for solid-phase protein chemistry and as effective micro devices for controlled release of protein drugs.

Alginate is a widely used polymer for immobilisation since it is highly biocompatible and easily biodegradable. Although satisfactory hydrolysis of oligosaccharides in soymilk has been achieved by using soluble purified α-galactosidases this process is costly and, in addition, the processed soymilk would contain foreign proteins.

In the present investigation, peanut α-galactosidase was immobilised in calcium alginate beads and used for hydrolysis reaction. The rationale of this system was to combine the low cost, nontoxicity and simplicity of alginate immobilisation with the high oligosaccharides bioconversion shown by peanut α-galactosidase.

The objective of this study is to:

a. Extract α-galactosidase enzyme from peanut.
b. Immobilise soluble enzyme in sodium alginate.
c. Study the kinetic properties of soluble and immobilised enzyme.
d. Study the operational stability of soluble and immobilised enzyme.
e. Investigate the application of soluble and immobilised enzyme for the hydrolysis of flatulence causing oligosaccharides in soymilk.
f. Study the extent of oligosaccharide removal in batch, repeated batch and continuous reactions.

2. LITERATURE REVIEW

2.1. Bovine milk and lactose intolerance

Bovine milk is a high quality staple food in many industrialised countries with strong dairy industries. The primary sugar in bovine milk is the disaccharide lactose, which is hydrolysed by the intestinal lactase to glucose and galactose. Most humans gradually lose the ability to synthesise lactase after infancy and with it the ability to digest lactose and such people acquire primary lactose intolerance or lactose malabsorption (Gilat, *et al.* 1972). Ingestion of a large dose of lactose through consumption of milk products causes diarrhoea, bloating, and flatulence in the majority of people with lactose intolerance (Cook & Dahlqvist 1968). Lactose intolerance affects millions of people all over the world and varying degrees of symptoms occur in patients, depending on the severity of their lactose intolerance and on the lactose load ingested.

Primary lactose intolerance can be controlled with strict adherence to lactose-soluble or lactose-reduced milk. Alternatively, gastrointestinal symptoms of lactose-intolerant people who consume milk products can be reduced with the use of commercially available lactase preparations that hydrolyse lactose to its components galactose and glucose (Gilat, *et al.* 1972). However, previous studies have shown that the gastric inactivation of the enzyme makes it less effective in alleviating lactose maldigestion (Onwulata, *et al.* 1989), and more expensive (Suarez, *et al.* 1995). Soymilk, which is lactose-soluble, is emerging as a potential alternative to bovine milk for lactose intolerance and more and more people are switching to soymilk or adding it to their diet.

2.2. Soymilk

Soymilk originated in China and is produced from soybeans **(***Glycine max***).** Soybean is rich in protein and has a well-balanced amino acid pattern (Smith & Circle 1972). The mature soybean is about 40 % protein, 35 % carbohydrate, 20 % lipid, and 5 % ash (Wolf & Cowan 1975). The composition of glucose, sucrose, raffinose, and stachyose in soluble carbohydrates of soybean is about 2.07 %, 58.01 %, 10.13 % and 29.80 %, respectively (Choung 2005).

Table 2.1 Composition and nutrient content of soymilk (100 grams)

Water (g)	93.3
Carbohydrate (g)	1.8
Fat (g)	1.9
Protein (Nx5.71)	2.8
Soluble Fiber (g)	1.1
Sodium (mg)	12
Calcium (mg)	4
Iron (mg)	0.58
Zinc (mg)	0.23
Thiamin (mg)	0.16
Riboflavin (mg)	0.07
Niacin (mg)	0.15
Vitamin B6 (mg)	0.04
Folacin (μg)	1.5
Sugar (g)	5
Kcal	33

Sucrose is the most prevalent oligosccharide in soybean soluble carbohydrates. It is also a rich source of isoflavones (Messina 1999) and soybean isoflavones have gained considerable attention in the past decade due to their potential

antioxidative and antihemolytic activities (Naim, *et al.*1976). The composition and nutrient content of soymilk are shown in Table 1 (USDA 1986).

Soymilk is the water extract of soybean and is produced by soaking soybeans in water, wet milling, and then extracting it with hot water, followed by separation of the milk from the cake. It is a nutritious beverage rich in high quality proteins and contains no cholesterol or lactose and only a small quantity of saturated fatty acids (Scalabrini, *et al.* 1998). It is a promising supplement to overcome existing protein-calorie malnutrition problems and is a low-cost nutritive supplement for lactose intolerant people (Greiner 1990). Soymilk contains 50 % more protein than cow's milk (Abiodun 1991). The food value of soymilk is limited because of the presence of flatulence-causing oligosaccharides raffinose (Figure 1) and stachyose (Figure 2) (Omosaiye, *et al.* 1978). Raffinose (Melitose; alpha-D-galactopyranosyl-(1-6)-alpha-D-glucopyranosyl-(1-2)-beta-D-fructofuranoside; $C_{18}H_{32}O_{16}$) is a trisaccharide, whereas, stachyose (Lupeose; beta-D-Fructofuranosyl-O-alpha-D-galactopyranosyl-(1-6)-O-alpha-D-galactopyranosyl-(1-6)-alpha-D-glucopyranoside; $C_{24}H_{48}O_{24}$) is a tetrasaccharide.

The enzyme α-galactosidase rapidly hydrolyses the 1, 6 linkages in raffinose and stachyose to give sucrose and galactose (Porter, *et al.* 1992). But humans lack the ability to synthesize this enzyme in their gastrointestinal tract and hence the oligosaccharides tend to remain unhydrolysed in the upper intestine of humans. However, the microflora in the lower intestine previously identified as gram-positive, spore forming anaerobic bacteria of *Clostridia* group are able to synthesize enzymes that ferment these oligosaccharides to produce CO_2, H_2, CH_4, H_2S and NH_3 (Singh, *et al.* 1993; Nnanna & Phillips 1990). The mixture of these gases produces flatulence, and intestinal discomfort that is specifically associated

with consumption of low molecular weight oligosaccharides (Phillips 1993; Steggerda, *et al.* 1966; Cristofaro, *et al.* 1974; Thananunkul, *et al.* 1976). Thus the reduction or removal of raffinose and stachyose in soymilk is a major factor in improving their nutritive value. Therefore, various attempts have been made to reduce the flatulence factors such as development of transgenic soybean plants with low raffinose family oligosaccharides (Suarez, *et al.* 1999), removal of these oligosaccharides from soymilk using solvents (Dey 1976), hydrolysis of raffinose family oligosaccharides by immobilized α-galactosidase (Mathew 1985), microbial fermentation by α-galactosidase secreting microorganisms (Tanaka, *et al.* 1976), and ultrafiltration of the aqueous fraction of soymilk (Suarez, *et al.* 1999).

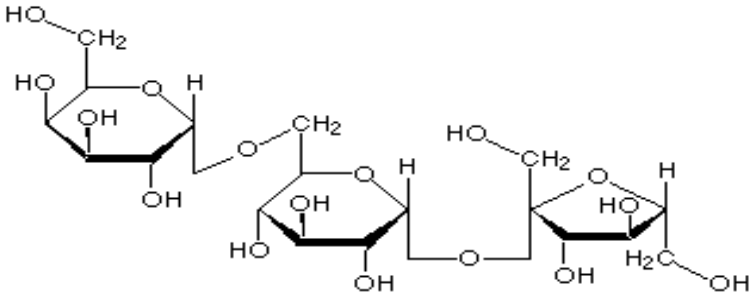

Figure 2.1 Chemical structure of Raffinose

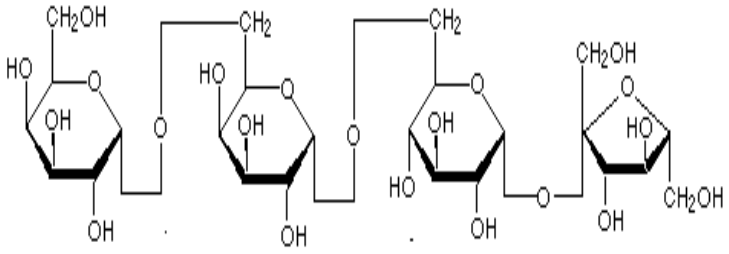

Figure 2.2 Chemical structure of Stachyose

2.3. Removal of flatulence-causing oligosaccharides

The elimination of flatulence-causing oligosaccharides is a challenging problem associated with the consumption of soymilk. Various methods such as ultrafiltration, reverse osmosis (Mok, *et al.* 1995), adsorption in activated charcoal (Kim, *et al.* 1994) have been used to remove the flatulence-causing oligosaccharides from soymilk. Rates of removal of oligosaccharides closely followed theoretical behaviour for a nonrejected solute during ultrafiltration and continuous diafiltration, and up to 96 % could be removed by a two-stage ultrafiltration process (Omosaiye 1978).

2.4. α-Galactosidase

The α galactosidase (α-gal) (α D-galactoside galactohydrolase) (EC.3.2.1.22) is an exoglycosidase that catalyses hydrolysis of terminal α-1-6 galactosidic bonds present in oligosaccharides (α-Galactosides) of raffinose family sugars such as melibiose, raffinose, stachyose and polymeric galactomannans and guar gum (Dey & Pridham 1972; Naumoff 2004). Moreover, it also hydrolyses glycoproteins and glycosphingolipids. α-Galactosidases have many potential biotechnological applications. These enzymes have different characteristics, such as, optimum pH, optimum temperature, ion requirements, inhibitors and side effects.

2.5. Occurrence of α-galactosidases

α-Galactosidases are widely distributed in nature, where it has been purified from a number of sources including plants, animals and microorganisms.

a) **α-Galactosidases from plants**

α-Galactosidase has been isolated and characterized from many plant sources (Dey & Pridham 1972). The enzyme is ubiquitous in legume seeds. It is present in sweet almond (Malhotra & Dey 1967), melon fruit (Zhifang & Arthur 1999),

endosperm of coconut (Balasubramaniam, *et al.* 1986), and sunflower seeds (Kim, *et al.* 2003). This enzyme has been isolated from the mature leaves of Cucurbita (*Cucurbita pepo*) (Thomas, *et al.* 1977) and from immature stalks of sugar cane (*Saccharum officinarum*) (Chinen, *et al.* 1981). Recently it has been isolated from tomato fruit (Feurtado, *et al.* 2001), grape flesh (Kang, *et al.* 2001) and cultured rice (Kim, *et al.* 2002). This enzyme is also present in peanuts (*Arachis hypogaea L.*) (Bryant & Rao 2001) and germinating seeds of coffee beans (Marraccini, *et al.* 2005).

b) **α-Galactosidases from animals**

In animals, α-galactosidase is first reported from snails (*Helix promatia*) (Bierry 1913). The presence of α-galactosidase from human spleen, placenta, plasma and liver (Bishop, *et al.* 1981; Dean, *et al.* 1979) has also been reported in literature. It is found in higher titers in rats especially in the cytoplasm of epithelial cells of Brunner's glands in the intestine (Suzuki, *et al.* 1972). Blood cells and bone marrow of some animals are also found to contain α-galactosidase (Monis, *et al.* 1967; Szmigielski 1966)

c) **α-Galactosidases from microorganisms**

Among microorganisms, α-galactosidase activity was first detected in brewers' yeast (Bau 1895; Fischer, *et al.* 1895). Subsequently, it was also detected in *Saccharomyces carlsbergensis* (Lazo, *et al.* 1977) and in *Aspergillus niger* (Scigelova 2000). Many bacteria have also been reported to contain α-galactosidase activity. Recently its presence is reported in extreme thermophilic eubacterium *Rhodothermus marinus* (Gomes, *et al.* 2000), marine bacterium *Pseudoalteromonas sp* (Bakunina, *et al.* 1998) and lactic acid bacterium *Carnobacterium piscicola* (Coombs, *et al.* 2001). Thermostable, neutral,

extracellular α -galactosidase with wide pH stability is produced by a newly isolated strain of *Bacillus stearothermophilus* grown on cheap agricultural residues at 60 ^{o}C, under submerged fermentation conditions (Gote, *et al.* 2004).

2.6. Significance of α-galactosidase

In plants and micro-organisms, the enzyme is involved in a variety of processes, most importantly in the hydrolysis of oligosaccharides such as raffinose and stachyose during the early germinative period, resulting in the liberation of soluble sugars, which may serve as a ready energy source for the growing plant (Dey, *et al.* 1972). In animals this enzyme hydrolyses galactolipids. The reduced activity of α-galactosidase results in Fabry's disease in humans, a deficiency resulting from the progressive accumulation of globotriaosylceramide and related glycosphingolipids. Affected patients have microvascular disease of the kidneys, heart, and brain (Feldt-Rasmussenet, *et al.* 2002). The enzyme also occurs in brain tissues with possible involvement in the hydrolysis of monogalactosyl diglycerides and digalactosyl diglycerides (Subba Rao, *et al.* 1970).

2.7. Crystal structure of α-galactosidase

The crystal structure of rice α-galactosidase has been determined recently. The structure of rice α-galactosidase consisted of a catalytic domain and a C-terminal domain. Catalytic domain has a (β/α) 8-barrel structure, and the C-terminal domain is made up of eight β-strands containing a Greek key motif (Figure 3) (Fujimoto, *et al.* 2003). The structure was determined in presence of D-galactose, providing a mode of substrate binding in detail.

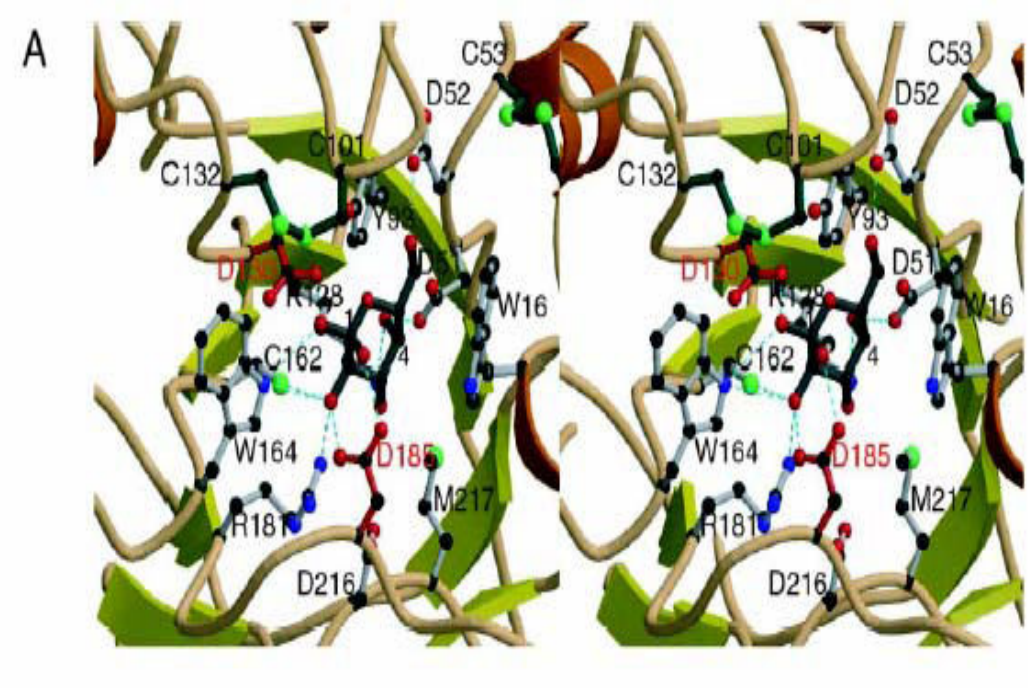

Figure 2.3 Stereo view of the ribbon model of rice α-galactosidase: The bound D-galactose, two catalytic residues, and two disulfide bonds are indicated by ball and stick drawings and shown in black, red, and green, respectively. Coordinating hydrogen bonds are shown in broken green lines (Fujimoto, *et al.* 2003).

2.8. Potential applications of α-galactosidases

Due to its ability to hydrolyse α-1, 6 linked D-galactosyl residues from galactose containing oligo and polysaccharides, α-galactosidase has many important applications in biotechnology and in food and feed processing. Transgalactosylation activity of α-galactosidases has frequently been used for the synthesis of new saccharides (Ajisaka & Fujimoto 1989). Currently α-galactosidase preparations are available as dietary supplements in humans' diets to reduce the problems related to flatulence. It is used in the processing of legume-based foods (Kotwal, *et al.* 1998), sugar beet molasses (Linden 1982), and guar gum processing (Cronin, *et al.* 2002). In the animal feed industry, it is used for increasing the digestibility of carbohydrates, proteins and fats in animal feed stuffs (Gdala, *et al.* 1997). In the paper and pulp industry, α-galactosidases could

enhance the bleaching effect of β-1, 4 mannanase on soft wood kraft pulp (Clarke, *et al.* 2000). In medicine, it plays a crucial role in the treatment of Fabry's disease (Breunig, *et al.* 2003) and for overcoming xenorejection for xenotransplantation (Stone, *et al.* 1998). The enzyme is also useful for structural analysis and for elucidation of the biological functions of complex natural compounds, since alpha-linked galactosyl units are constituents of many oligosaccharides, polysaccharides, glycoproteins and glycolipids (Zaprometova, *et al.* 1990; Kusakabe 1990).

2.9. Enzyme immobilisation

Almost 95 % of the commercial enzymes are available in a soluble form. The application of soluble enzymes in the biotechnological and food industry field is not always possible. As it is readily dispersed in the solution, it is often both costly and technically difficult to recover an active form of the enzyme from product mixtures when the reaction of interest is completed. The freedom of movement of an enzyme is greatly restricted by immobilisation of enzymes on insoluble polymer supports or carriers. The solid phase containing the enzyme is easily recovered from the product mixture. Use of immobilised enzymes also makes it possible to conduct continuous reaction, thereby obtain more products per unit of enzyme employed. The high product yield minimises downstream processing costs and the environmental impact of the process.

There are several methods of enzyme immobilisation (Bornscheuer 2003). Effective methods of immobilisation include physical adsorption onto a solid support, encapsulation, cross-linking, and covalent binding. A key requirement of enzyme immobilisation is attachment without sacrificing enzyme activity. The

first consideration is to decide on the support material, then the main method of immobilisation, taking into account the intended use and application.

Among the methods of immobilisation, entrapment in ionic gels is one of the simplest, cheapest and mildest immobilisation methods (Busto 1998; Schneider 1991; Manjon, *et al.*1995). Natural polymers, especially polysaccharides, have been widely used because of their unique advantages such as non-toxicity, biocompatibility, biodegradability, and abundant favourable properties. Formation of spherical gel beads is spontaneous and results from ionic network formation, which is based on anionic cross-linking of polyelectrolyte such as alginate, carrageenan, chitosan, and polygalacturonic acid with multivalent ions (Vasir, *et al.* 2003; Kumar 2000).

Alginate is by far the most widely used polymer for immobilisation and microencapsulation technologies (Funduenanu, *et al.* 1999; Velten, *et al.* 1999). Alginate is a seaweed extract composed of chains of alternating α-L-guluronic acid and α-D-mannuronic acid residues (Sriamornsak 1998). Alginate supports are usually made by cross-linking the carboxyl group of the α-L-guluronic acid with a solution of a cationic cross linker such as calcium chloride, barium chloride (Draget 1997; Smidsrod, *et al.* 1990). Alginate matrices cross-linked with Ca^{2+} ions, however, are unstable in the physiological environment or in common buffer solutions with high concentration of phosphate and citrate ions that can extract Ca^{2+} from the alginate and liquefy the system.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Enzyme

α-Galactosidase enzyme used for hydrolysis of soymilk was isolated from peanuts purchased from the local market (Hamilton, New Zealand).

3.1.2. Chemicals

All the chemicals used in this study were of analytical grade. p-Nitrophenyl-α-D-galactopyranoside (p-NPG), sodium phosphate (monobasic and dibasic), p-nitrophenol (p-NP), sodium alginate, raffinose, stachyose, and Bradford reagent were procured from Sigma Chemical Co. (MO, USA). Calcium Chloride was obtained from BDH Laboratory Supplies (New Zealand). Phenol, silver nitrate, and sodium hydroxide were obtained from Unilab (New Zealand). All reagents were prepared using distilled water.

3.2. Methods

3.2.1. Soluble enzyme preparation

Soluble enzyme preparations were made according to the following procedures.

a. Extraction with acetone

One hundred grams of the decorticated peanuts were homogenised with 100 ml of cold (4 ^{o}C) acetone using a homogeniser (Silverson L4RT). After decanting the acetone, the procedure was repeated twice using 75 ml of cold acetone. The ground sample was combined with the decanted acetone and the mixture was centrifuged at 4000 rpm for 10 min at 4 °C. The acetone was discarded and the precipitate was washed twice with cold acetone, air-dried and stored at - 20 °C. The preparation is referred to as the acetone powder (Bryant & Rao 2000).

b. Extraction with sodium phosphate buffer

One hundred grams of peanuts were crushed with 200 ml of 0.1 M sodium phosphate buffer at pH 6.5, filtered and centrifuged at 4000 rpm for 15 min to obtain raw enzymatic extract. The slurry obtained was air-dried.

c. Ammonium sulphate precipitation

Twenty-five grams of acetone powder were extracted with 100 ml of cold 0.1 M acetate buffer (pH 5.0) using a magnetic stirrer for 1 hour. After decanting the buffer, the residue was re-extracted with additional 100 ml of acetate buffer and the combined extracts were centrifuged at 4000 rpm for 10 min at 4 ºC**.** The buffer extract was filtered and solid ammonium sulphate was added slowly to the filtrate with stirring until saturation (Bryant & Rao 2000). The solution was filtered and the precipitate obtained was air-dried.

3.2.2. Entrapment of soluble α-galactosidase in calcium alginate beads

A solution of 3 % (w/v) sodium alginate was mixed with 30 mg soluble α-galactosidase. The alginate-enzyme mixture was made into beads by dropping the alginate solution into 0.2 M $CaCl_2$ as cross-linking agent. The distance from the drain tube to the surface of the calcium chloride solution was approximately 20 cm. The beads (2 mm diameter) were allowed to remain in the calcium chloride solution overnight. After immobilisation, the beads were removed, washed with distilled water and stored at 4 ^{o}C in 0.1 M phosphate buffer (pH 6.5) until further use.

3.2.3. Protein determination

The protein content of the soluble enzyme was determined according to the method of Bradford (1976) using bovine serum albumin (BSA) as a reference protein.

3.2.4. Assay of α-galactosidase activity using sodium phosphate buffer

The activity of the soluble and the immobilised α-galactosidase were determined according to the procedure of Bryant & Rao (2000) by adding 10 ml of 0.1 M sodium phosphate buffer (pH 6.5) to 30 mg (protein content = 21.6 mg) of the enzyme extract. After vortexing for 2 min, 1250 µmol of the solution were removed and warmed to 37 ºC for 5 min in a water bath. 250 µl of 0.25 mM solution of p-NPG in distilled water was then added and the mixture was incubated at 37 ºC for 10 min. The reaction was terminated by adding 250 µl of a solution of sodium carbonate (5 % w/v) in water and the liberated p-NP was determined spectrophotometerically at 400 nm. All experiments were performed at least in triplicate and the results are presented as their mean values.

3.2.5. Assay of α-galactosidase activity using McIlvain buffer

The enzyme activity assay was also carried out by dissolving the substrate in McIlvain (phosphate-citrate) buffer in order to identify a suitable buffer (Bryant & Rao 2000). 10 ml of 0.1 M McIlvain (phosphate–citrate) buffer (pH 6.5) was added to 30 mg (protein content = 21.6 mg) of acetone powder. After vortexing for 2 min, 1250 µmol were removed and warmed to 37 ºC for 5 min in a water bath. 250 µl of 0.25 mM p-NPG in distilled water was then added and the mixture was incubated at 37 ºC for 10 min. The reaction was terminated by adding 250 µl of a solution of sodium carbonate (5 % w/v) in water and the liberated p-NP was determined spectrophotometerically at 400 nm. All experiments were performed at least in triplicate and the results are presented as their mean values.

One unit of α-galactosidase activity (U) is defined as the amount of enzyme liberating one µmol of p-NP in one minute under the conditions of assay. The amount of p-NP released was calculated from an appropriate calibration curve

at 400 nm. The specific activity of soluble α-galactosidase was calculated by dividing the enzyme activity (U) by the amount of protein in the enzyme. The specific activity of immobilised α-galactosidase was calculated by dividing the enzyme activity (U) by the amount of protein bound to the beads. The specific activity is expressed as U/mg protein. Residual activity of soluble and immobilised enzyme was determined by dividing the specific activity (U) by the highest specific activity.

3.2.6. Activity yield

Activity yield of immobilisation is defined as the percentage of the specific activity of the immobilised α-galactosidase with respect to the specific activity of the soluble α-galactosidase and is calculated as follows.

Activity yield (%) = $B/A \times 100$, where A is the total activity of enzyme added in the initial immobilization solution; and B is the activity of the immobilized enzyme.

3.2.7. Kinetic investigations

Kinetic parameters (K_m and V_{max}) of soluble and immobilised enzyme were determined by Michaelis-Menten enzyme kinetics by using increasing concentrations (0.025 mM - 0.15 mM) of substrate (p-NPG) at 37 ^{o}C. The reaction mixture consisted of 1250 µl p-NPG in 0.1 M phosphate buffer (pH 6.5). The reaction was initiated by adding 30 mg soluble enzyme (protein content = 21.6 mg) into the reaction mixture. After 10 min, the reaction was terminated by adding 250 µl of a solution of sodium carbonate (5 % w/v) in water and the liberated p-NP was determined spectrophotometerically at 400 nm. The K_m and V_{max} were calculated from the double reciprocal plots (Lineweaver-Burk plot) of Michaelis-Menten equation. For determining K_m and V_{max} of immobilised enzyme, beads containing 30 mg soluble enzyme (protein content = 21.6 mg)

were used. All experiments were performed in triplicate and the results are presented as their mean values.

3.2.8. Operational stability of soluble and immobilised α-galactosidase

3.2.8.1. pH stability

The pH stability was determined by calculating the residual activity of soluble and immobilised enzyme after incubating at varying pH (3-8) and at 37 ^{o}C. The reaction mixture consisted of 1250 µl p-NPG in 0.1 M phosphate buffer. The reaction was initiated by adding 30 mg soluble enzyme (protein content = 21.6 mg) into the reaction mixture. After 10 min, the reaction was terminated by adding 250 µl of a solution of sodium carbonate (5 % w/v) in water and the liberated p-NP was determined spectrophotometerically at 400 nm.

3.2.8.2. Thermal stability

The thermal stability was determined by calculating the residual activity of soluble and immobilised enzymes after incubating at temperatures varying from 20 to 70 °C. The reaction mixture consisted of 1250 µl p-NPG in 0.1 M phosphate buffer (pH 6.5). The reaction was initiated by adding 30 mg soluble enzyme (protein content = 21.6 mg) into the reaction mixture. After 10 min, the reaction was terminated by adding 250 µl of a solution of sodium carbonate (5 % w/v) in water and the liberated p-NP was determined spectrophotometerically at 400 nm.

3.2.8.3. Reusability

The reusability of immobilised α-galactosidase was determined by repeatedly using the enzyme in p-NPG hydrolysis. The retention of the immobilised enzyme activity was determined as described in the activity assay of α-galactosidase in Section 3.2.4. After each reaction run, the enzyme immobilised alginate beads were removed from the reaction mixture and washed with 0.1 M sodium

phosphate buffer to remove any residual substrate within the beads. The beads were then reintroduced into fresh reaction medium. Substrate was then added and the solution incubated at 37 ºC for 10 min.

3.2.8.4. Storage stability

The residual activity of soluble and immobilised α-galactosidase after storage at 5° C in 0.1 M sodium phosphate buffer (pH 6.5) was measured in a batch operation mode and assayed for specific activity every five days.

3.2.9. Soymilk preparation

Whole soybeans obtained from the local market were washed and soaked in distilled water overnight. The swollen soybeans were dehulled and then suspended in warm water and homogenised (Silverson L4RT Homogeniser). The ratio of soaked soybean to water used was 1:10 (w/v). The slurry was filtered through double-layered cheesecloth and the filtrate was centrifuged at 4000 rpm for 20 min. The supernatant was transferred into glass bottles and stored in a refrigerator (4 °C) until use.

3.2.10. Determination of oligosaccharides in soymilk

3.2.10.1. Thin Layer Chromatography (TLC)

The amount of raffinose and stachyose in soymilk was estimated by using TLC. TLC was performed on aluminium sheets (20 x 20cm) coated with silica gel G260 (Merck, Germany). Ten micro litres of soymilk was applied to thin layer chromatography (TLC) plates. The plates were developed with different chromatography solvents for optimising the solvent. The solvents used were n-propanol-ethyl acetate-water (7:2:1) (Tanaka, *et al.* 1976), 1-butanol-ethanol-water (5:5:3) (Kim & Sakano 1996), ethyl acetate-2-propanol-water (6.5:2.85:1.15), and 1-butanol-2-propanol-water (1:7:2) (Smith 1958), n-butanol-

ethylacetate-isopropanol-water (35:10:60:30) (Jones, *et al.* 1999), n-butanol-ethanol-ammonia-water (8:1:1:2) (Onigbinde & Akinyele 1983). After the run, the plates were dried and the sugars were spotted by spraying with 1 % α-naphthol in absolute alcohol containing 10% orthophosphoric acid (Albon, *et al.* 1950). The other developing reagent used was 10 % silver nitrate in acetone (Onyesom, *et al.* 2005). The spots were identified based on retention factor.

3.2.10.2. Quantitative analysis

The identified spots in TLC were carefully cut out and each spot was separately soaked in 2 ml of distilled water for total dissolution of the sugars. 0.1 ml of 5 % phenol was added to each dissolved sugar followed by rapid addition of 0.5 ml concentrated sulphuric acid. The tubes were then placed in a water bath to cool for 20 min. The absorbance was read at 490 nm and the corresponding concentration was determined from a standard curve previously prepared from each reference sugar (Onyesom, *et al.* 2005).

3.2.11. Enzymatic hydrolysis of soymilk

3.2.11.1. Batch reaction

Batch reaction was performed for soluble and immobilised enzymes at different incubation periods. For the reaction involving soluble enzyme, 30 mg of the soluble enzyme (protein content = 21.6 mg) was added to 60 ml of soymilk in cotton plugged conical flasks (100 ml). For the reaction involving immobilised enzyme, calcium alginate beads containing 30 mg soluble enzyme (protein content = 21.6 mg) was added to 60 ml soymilk. The hydrolysis reaction was carried out at 55 ºC in an incubator shaker at 200 rpm for different incubation periods of 2, 4, 6, 8, 10 and12 h. After the incubation period an aliquot of the reaction mixture was withdrawn and concentrations of raffinose and stachyose

were determined. For the reaction involving soluble enzyme, reaction mixtures were kept in a boiling water bath for 10 min to arrest the enzyme reaction before determining the oligosaccharides content.

3.2.11.2. Repeated batch reaction

To establish the stability of the oligosaccharide degradation by the immobilised enzyme, repeated batch experiments were also carried out. 60 ml of soymilk with immobilised enzyme beads containing 30 mg soluble enzyme (protein content = 21.6 mg) were taken in a 100 ml conical flask and kept in an incubator shaker (200 rpm) at 55 ºC. After every 4 h of incubation, an aliquot of the soymilk was taken out and the oligosaccharide concentration determined. The beads were then separated by filtration, washed with distilled water, and transferred into another new batch of soymilk (60 ml) for 4 h incubation at 55 ºC.

3.2.11.3. Continuous reaction

The experimental set-up for the continuous hydrolysis of raffinose and stachyose in soymilk is shown in Figure 4. Continuous reaction was carried out in a glass column (21.5 cm x 2 cm diameter) packed with the beads containing immobilised enzyme. Soymilk feed solution at 55 ^{o}C was introduced at the bottom of the column at a flow rate of 12 ml/h using a micro pump (Biolab Scientific Limited, US). The outlet stream was returned to the feed reservoir. Samples were collected at regular intervals and analysed for raffinose and stachyose in TLC.

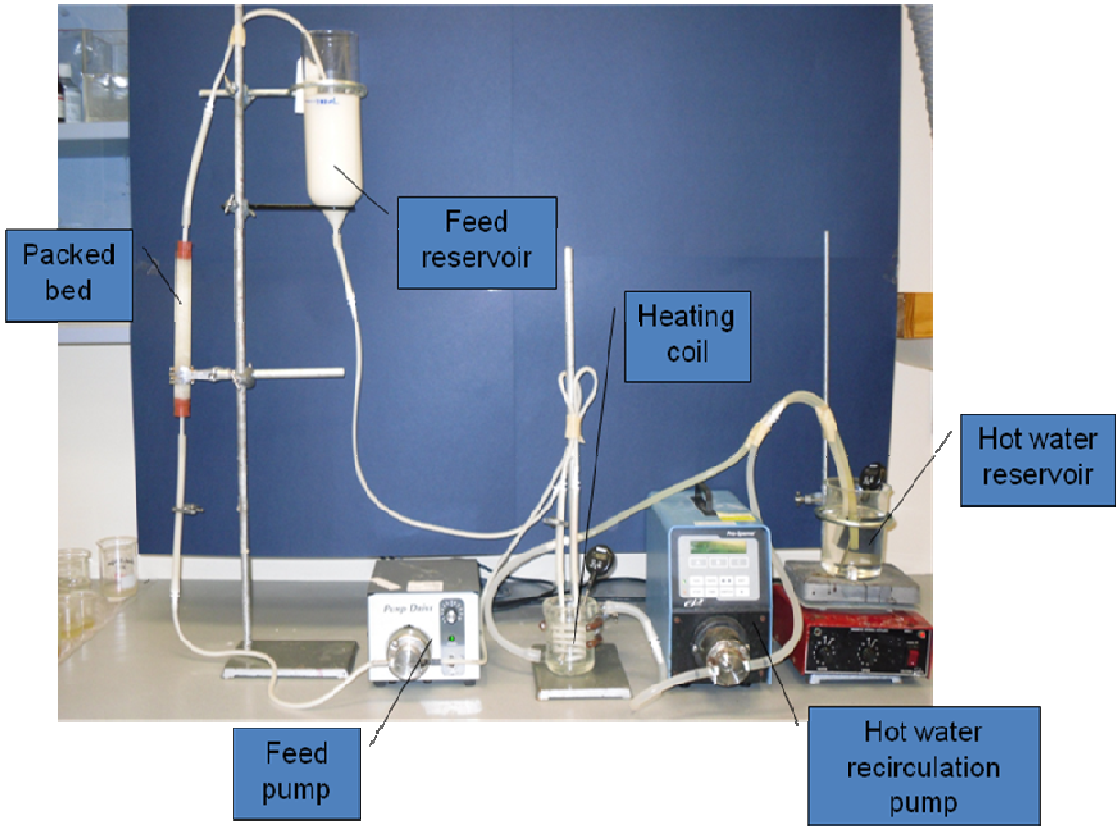

Figure 3.1 Continuous packed bed reactor

4. RESULTS AND DISCUSSION

4.1. Enzyme extraction and activity

Peanut (*Arachis hypogaea L.*), one of the world's greatest sources of edible vegetable oil, has not been explored with respect to its α-galactosidase activity. Peanut has shown to be a cheap and potential source of α-galactosidase. In this study, we investigated the application of soluble and immobilised α-galactosidase obtained from peanuts for the hydrolysis of flatulence causing oligosaccharides in soymilk and studied the extent of oligosaccharide removal in batch, repeated batch and continuous reactors.

The specific activities of soluble preparations of peanut α-galactosidase obtained by various methods described in Materials and Methods are shown in Table 4.1. The specific activity (U/mg protein) of soluble preparation of α-

galactosidase ranged from 0.022 to 0.06. The enzyme fraction precipitated with phosphate buffer showed the highest specific activity (0.06 U/mg protein). Bryant and Rao (2000) reported a specific activity of 0.04 U/mg protein for peanut α-galactosidase obtained with 0.1 M cold sodium phosphate buffer (pH 6.5) extraction followed by 1-fold purification using dialysis. In this study, the specific activity of 0.06 U/mg protein obtained for soluble enzyme was higher when compared to that obtained by Bryant and Rao (2000). Peanut α-galactosidase was immobilised in sodium alginate by entrapment. The specific activity of the immobilised α-galactosidase was found to be 0.08 U/mg protein. There was an increase of 33 % in the specific activity of the soluble enzyme as a result of immobilisation.

Table 4.1 Specific activities of soluble α-galactosidase from peanuts

Extraction method	Activity (U)	Specific activity of soluble enzyme (U/mg protein)
Phosphate buffer extract	1.77	0.06
Ammonium sulphate fraction	0.90	0.03
Acetone extract	0.90	0.03
McIlvain (phosphate–citrate) buffer	0.65	0.02

The activity yield, defined as the percentage of specific activity of the immobilised enzyme to that of the soluble enzyme, is a key parameter

representing the general output of the efficiency of the immobilisation process. The activity yield of α-galactosidase immobilised in calcium alginate beads was 33%.

4.2. pH stability of immobilised α-galactosidase

The variation of residual activity of immobilised α-galactosidase with pH was compared with that of the soluble enzyme and the results are shown in Figure 4.1.

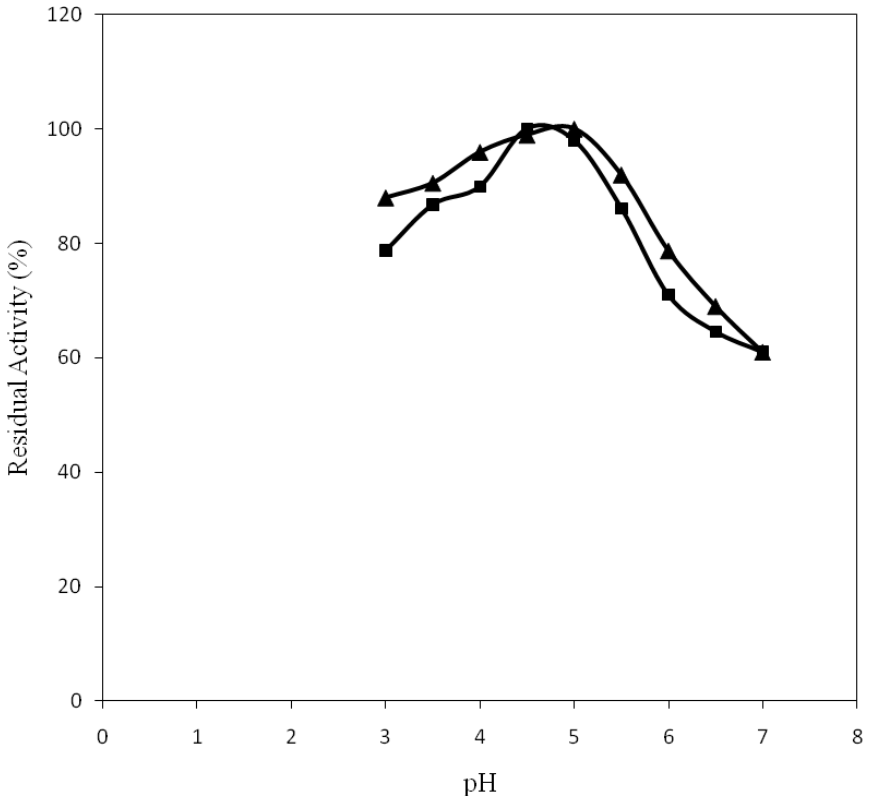

Figure 4.1 Effect of pH on (■) soluble and (▲) immobilised α-galactosidase at 37 ^{o}C

The soluble enzyme exhibited 79 % residual activity at pH 3.0 and 61 % at pH 7.0, while the immobilized enzyme exhibited 88 % residual activity at pH 3.0 and 61 % at pH 7.0. The optimum pH for soluble and immobilised α-galactosidase was found to be 4.5 and 5, respectively. Prashanth & Mulimani (2005) obtained an optimum pH of 5.0 and 5.5, respectively, for purified soluble α-galactosidase

from *Aspergillus awamori* and *Aspergillus saitoi* that was used for the hydrolysis of oligosaccharides in soymilk. Furthermore, the immobilised α-galactosidase was slightly more stable at acidic pH (4.5). The higher residual activities observed in the acidic range for α-galactosidase immobilised in calcium alginate beads indicated that the immobilisation process enhanced the enzyme stability in the acidic range (Figure 4.1)

4.3. Temperature stability of immobilised α-galactosidase

The temperature optimum of the soluble and alginate immobilised α-galactosidase was determined by assaying the enzyme activity at varying temperatures (25 - 70 ^{o}C) and the results are shown in Figure 4.2.

The residual activities of the soluble and the immobilised enzymes increased with temperature. The immobilized enzyme exhibited higher residual activities indicating that the immobilisation process enhanced the thermal stability of the enzyme. The maximum residual activity of immobilized enzyme was obtained at 55 ^{o}C compared to 50 ^{o}C for the soluble enzyme. Optimum temperature for the activity of immobilized enzyme shifted to a higher value (Figure 4.2).

The soluble enzyme exhibited 70.9 % residual activity at 70 ^{o}C, whereas the immobilised enzyme had around 89.0 % activity. Immobilisation of enzyme in alginate beads caused an increase in enzyme rigidity, which is commonly reflected by an increase in stability towards denaturation by raising the temperature (Abdel-Naby, 1993). The decrease in activity of the enzyme at higher temperatures may be due to the thermal degradation.

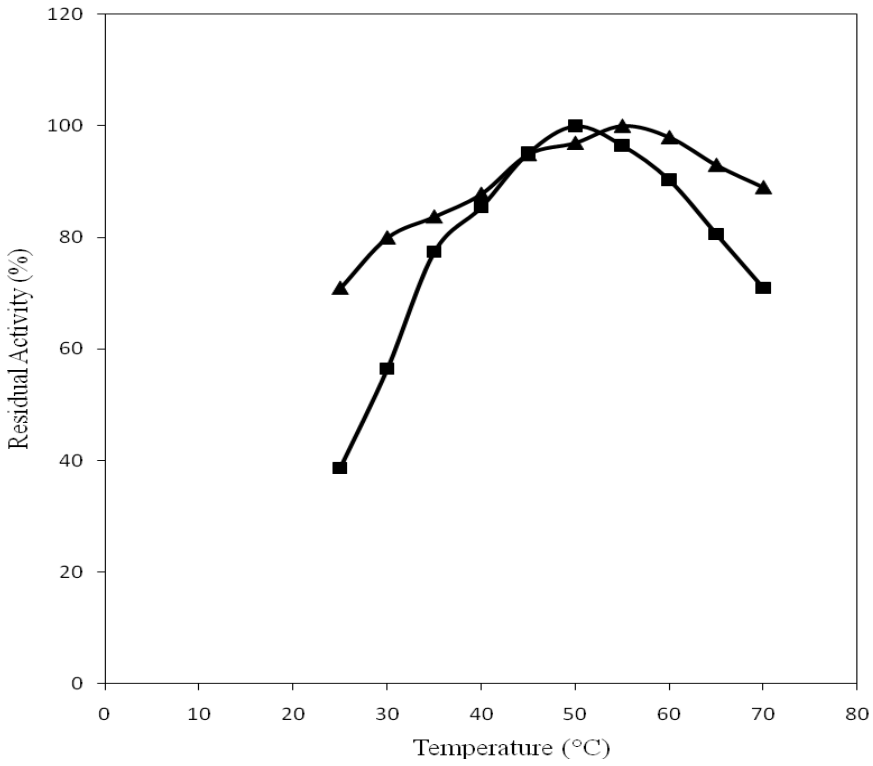

Figure 4.2 Effect of temperature on (■) soluble and (▲) immobilised α-galactosidase at pH 6.5

4.4. Reusability of immobilised α-galactosidase

When comparing the performance of immobilised enzymes intended for industrial use, characterization of their operational stabilities is very important. The reusability and storage stability of immobilised α-galactosidase in the current study was evaluated in a batch process. Figure 4.3 shows the effect of repeated use on residual activity of calcium alginate immobilised α-galactosidase. No significant activity loss on immobilised enzyme was observed up to three repeated uses. This brings an advantage over soluble enzyme use. The immobilised enzyme retained 96 % residual activity after three uses. Thereafter the residual activity gradually decreased. The decrease in activity of the immobilised enzyme upon prolonged use may be attributed to the leakage of the enzyme from alginate beads.

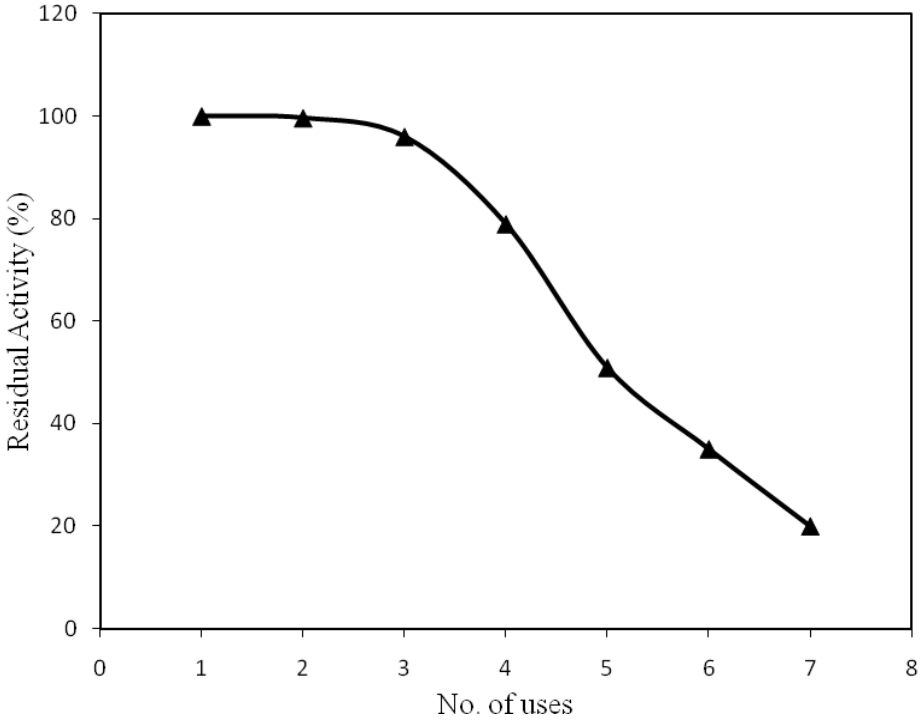

Figure 4.3 Reusability of immobilised α-galactosidase at pH 6.5 and 37 ºC

4.5. Storage stability of immobilised α-galactosidase

The storage stability of the immobilised α-galactosidase was studied by storing the enzyme in a phosphate buffer (pH 6.5) and in batch reactions. The residual activities obtained are shown in Figure 4.4. The immobilised enzyme did not exhibit considerable activity loss up to 30 days storage at 4 ºC, but the activity decreased after 30 days. The decrease in activity is explained as time dependent natural loss, and as the leakage of enzyme from the alginate, even at high concentrations, which gradually leads to loss of the enzyme activity and this can be prevented to a significant degree by the immobilisation process.

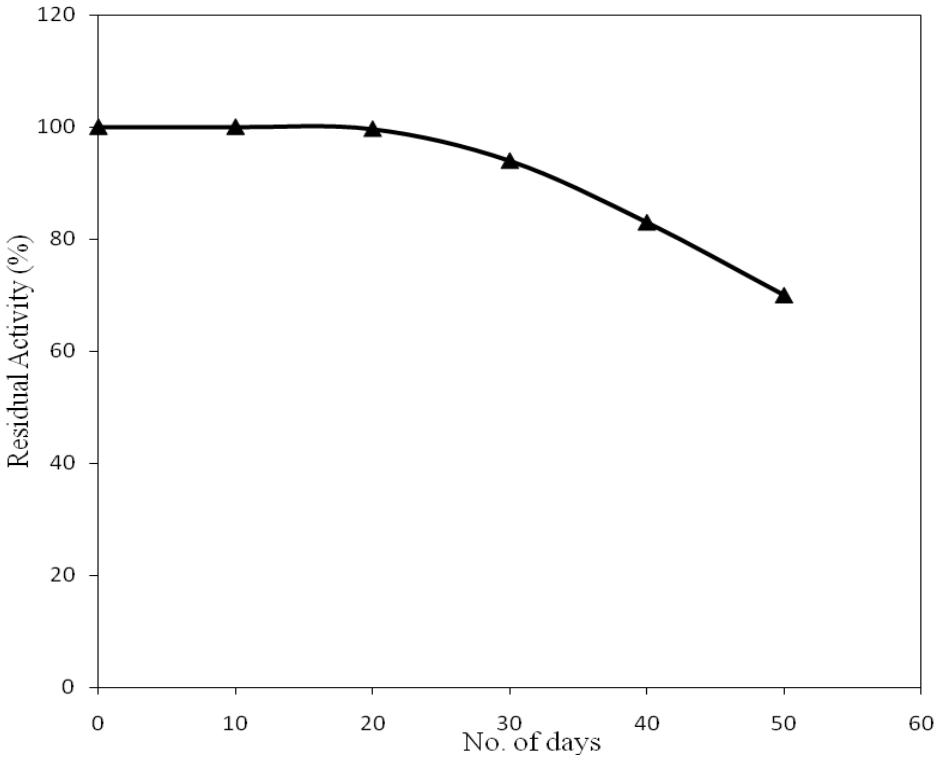

Figure 4.4 The storage stability of immobilised enzyme

4.6. Kinetic studies

Kinetics of soluble and immobilised α-galactosidase was investigated at various concentrations (0.02 mM - 0.15 mM) of p-NPG as substrate at 37 ^{o}C. Apparent K_m and V_{max} were calculated from Lineweaver-Burk (1/v vs. 1/s). The results obtained are shown in Table 4.2. As expected, the V_{max} of the enzyme increased upon immobilisation on calcium alginate beads. But, α-galactosidase immobilised alginate beads exhibited apparent K_m value, which was about two fold higher than that of soluble α-galactosidase. Bodalo, *et al.* (1991) reported that K_m value increased and V_{max} decreased upon immobilisation of α-galactosidase in alginate. The increase in K_m after immobilisation may be partially due to mass transfer resistance of the substrate into the alginate beads. Low diffusion is a characteristic shown by alginate immobilisation system. The alginate network, due to its high

degree of crosslinking limits the permeation rate of substrate and product (Florido, *et al.* 2001). The comparatively low V_{max} obtained may be due to the use of the crude nature of the enzyme and could be enhanced through proper purification of the enzyme.

Table 4.2 Kinetic parameters for soluble and immobilised α-galactosidase

Type of enzyme	V_{max} (μmol/L)	K_m (mM)
Soluble enzyme	0.00032	0.0134
Immobilised enzyme	0.0017	0.027

4.7. Enzymatic treatment of soymilk

4.7.1. Batch reaction

The results of the batch hydrolysis of raffinose and stachyose in soymilk by soluble and immobilised α-galactosidase are shown in Figures 4.5 and 4.6. For batch hydrolysis, soymilk was incubated with soluble and immobilised enzyme for different incubation periods, i.e., 2, 4, 8, and 12 h.

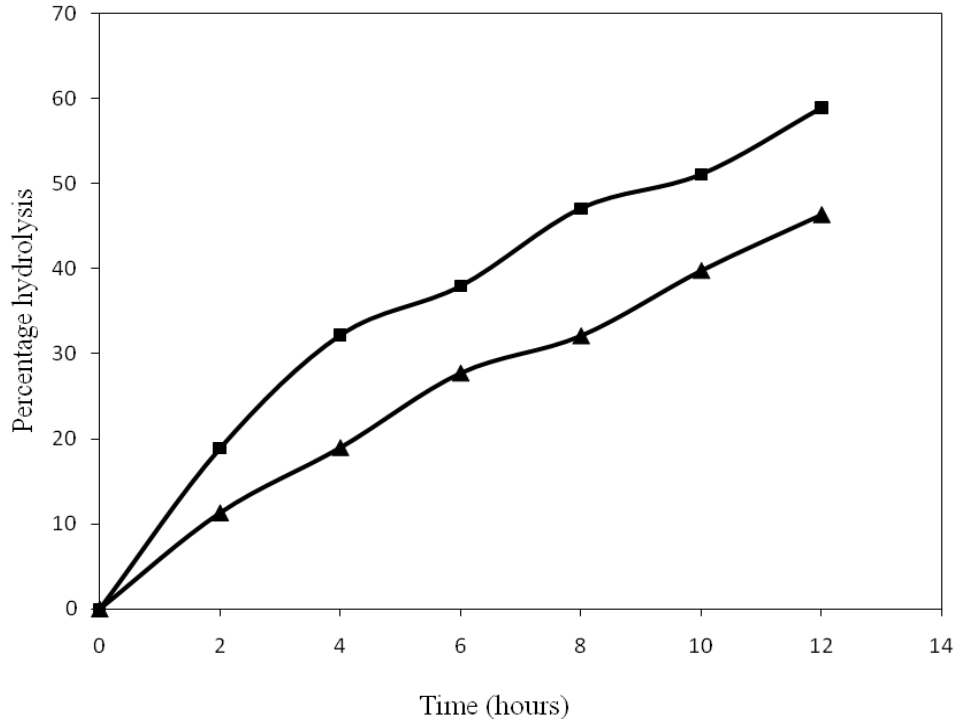

Figure 4.5 Time course of batch hydrolysis of raffinose in soymilk using (■) soluble and (▲) immobilised α-galactosidase

After 12 h incubation the use of soluble α-galactosidase led to 58.9 % raffinose reduction, whereas the use of immobilised α-galactosidase resulted in 46.35 % reduction in raffinose. About 76.79 % of stachyose in soymilk was hydrolysed by the immobilised enzyme, whereas soluble enzyme hydrolysed 80.55 % of stachyose. The soluble enzyme showed better hydrolysis efficiency than the immobilised enzyme. This was explained by Abdel-Naby, *et al.* (1999) as the diffusion limitation (i.e. resistance of substrate to diffuse into the immobilisation matrix and resistance of the products to diffuse out). A lower hydrolysis of raffinose and stachyose in soymilk was obtained in this study when compared with the results reported in literature. This may be due to the use of the crude enzyme used in this study. The percentage hydrolysis could be enhanced by using purified enzyme for immobilisation. However, the cost of purification would

increase the overall process cost and make the process less attractive commercially.

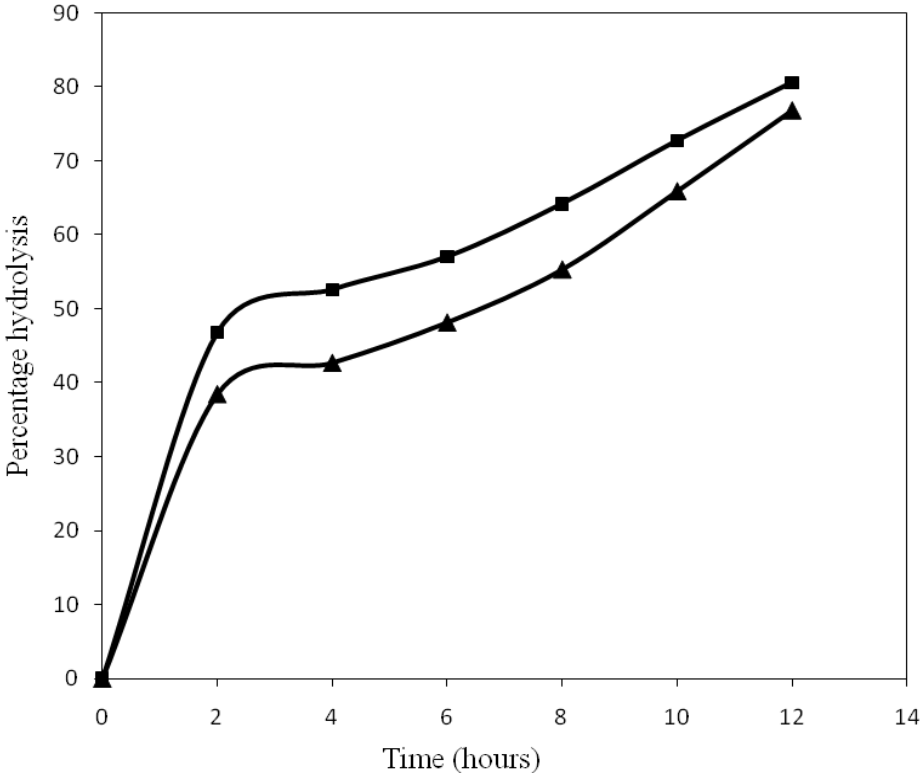

Figure 4.6 Time course of batch hydrolysis of stachyose in soymilk using (■) soluble and (▲) immobilised α-galactosidase

Decreasing the K_m of the immobilised enzyme by modifying the porosity of the alginates beads could be an alternative way to reduce the mass transfer limitation and to obtain higher hydrolysis rates. The low hydrolysis rate may be due to product inhibition (Sugimoto 1970).

By using *α*-galactosidase from *Mortierella vinacea* entrapped in polyacrylamide gel, Tanaka, *et al.* (1976) obtained 50 % hydrolysis of raffinose and stachyose in soymilk. Similarly, by using soluble α-galactosidase from *Gibberella fujikuroi*, Mulimani (1995) obtained 71 % reduction of flatulence factors (raffinose + stachyose) in soymilk after 12 h incubation.

4.7.2. Repeated batch reaction

The efficiency of the immobilised α-galactosidase for the removal of flatulence-causing oligosaccharides was evaluated in a repeated batch process. Hydrolysis rates were calculated after every 4 h of incubation of soymilk with the immobilised enzyme.

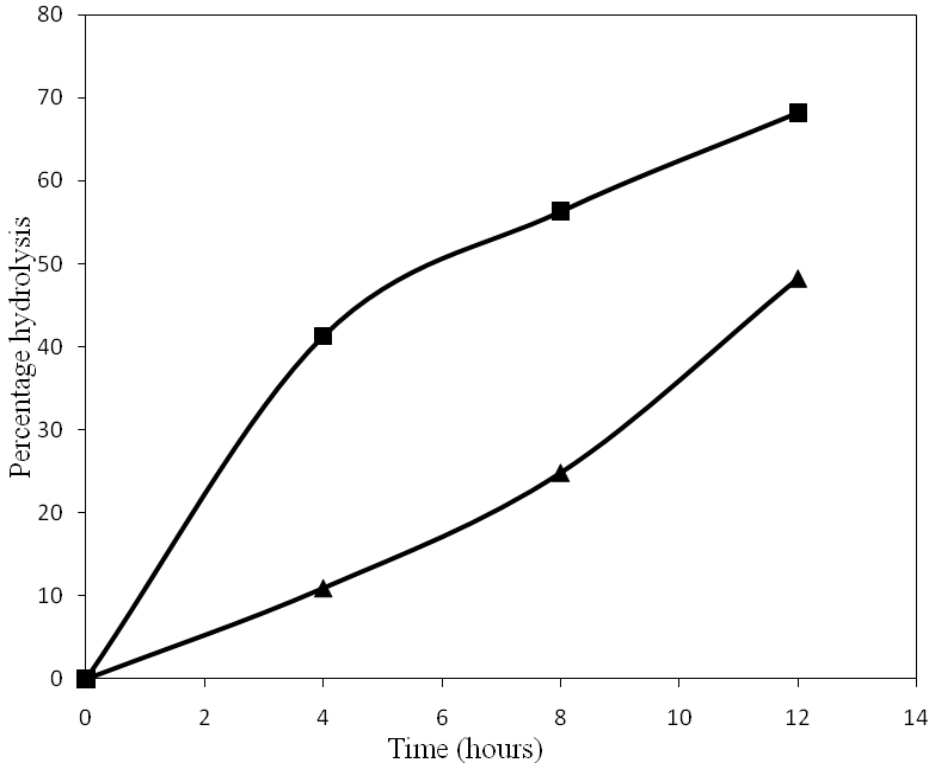

Figure 4.7 Time course of repeated batch hydrolysis of (■) stachyose and (▲) raffinose in soymilk using immobilised α-galactosidase

The results shown in Figure 4.7 indicated that the hydrolytic activity of the immobilised enzyme was retained after repeated use. Thus, 68.2 % stachyose reduction and 48.17 % raffinose reduction was obtained with the immobilised enzyme after three repeated runs. This proves the ability of the immobilised enzyme for potential large-scale use for the reduction of flatulence-causing sugars in soymilk.

4.7.3. Continuous reaction

The large-scale use of immobilised α-galactosidase was investigated in a packed bed reactor at a flow rate of 11.9 ml/min and the results obtained are shown in Figure 4.8.

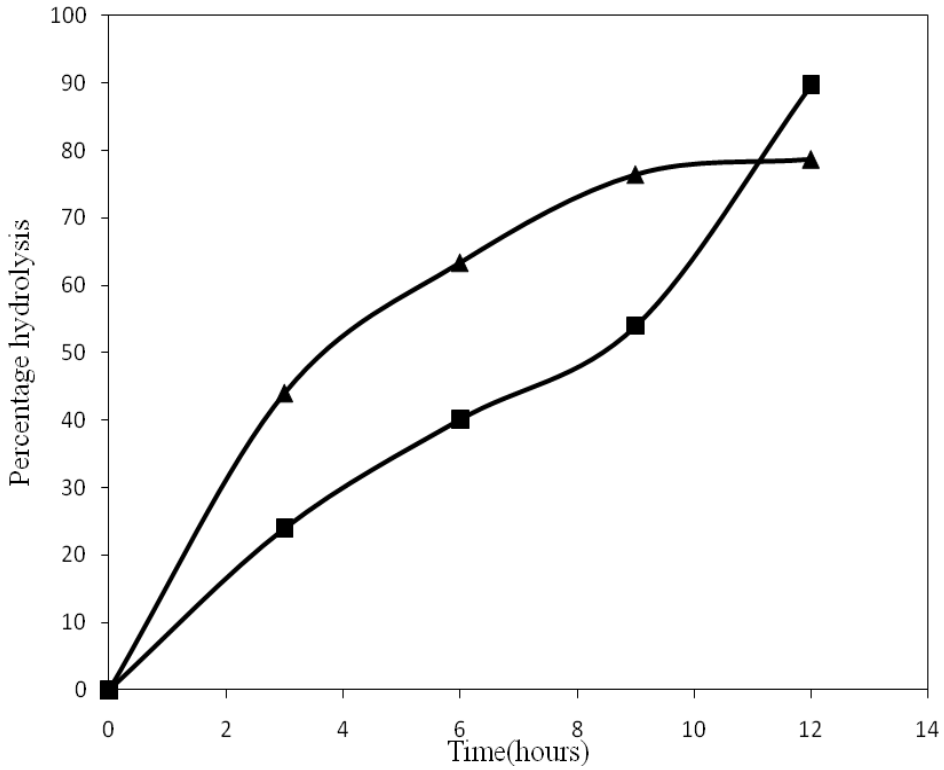

Figure 4.8 Time course of (■) stachyose and (▲) raffinose hydrolysis using immobilised α-galactosidase in a packed bed reactor

After 12 h of continuous reaction 89.78 % of stachyose and 78.71 % of raffinose in soymilk was hydrolysed. To our knowledge this is the first investigation concerning the use of alginate immobilised α-galactosidase in a packed bed reactor for the hydrolysis of flatulence-causing oligosaccharides in soymilk.

5. CONCLUSION

Enzymatic hydrolysis of flatulence causing oligosaccharide in soymilk has been reported in literature. This study was done to explore the potential of peanut α-galactosidase for large-scale hydrolysis of the flatulence causing oligosaccharides in soymilk. The specific activity of peanut α-galactosidase seems to be low but it was apparently due to the use of soluble enzyme without any purification process. To our knowledge the use of a packed bed reactor for the continuous hydrolysis of flatulence-causing oligosaccharides in soymilk has not been attempted before. This provides the feasibility of using the enzyme in a large-scale process by remarkably reducing the production cost. α-Galactosidase was immobilised in alginate beads, which is a simple and cheap process with durable enzyme activity. Eventhough other polymeric materials are readily available for immobilisation of α-galactosidase calcium alginate is an excellent choice since it is safe for human consumption. Immobilised α-galactosidase shows high optimum temperature and a wide pH range. α-Galactosidase shows activity even at pH 7, which enables it for use in soymilk hydrolysis. The pH of soymilk was in the range of 6.0 to 6.5. Raffinose and stachyose contents of soymilk vary with the variety of soybeans. But it can be said that the raffinose and stachyose in soluble carbohydrates is 10.13 % and 29.80 %, respectively (Choung 2005). In previous research, scientists had obtained different values for raffinose and stachyose content in soymilk using the same TLC method as used in this study. Mulimani (1995) reported 1.95 % raffinose and 6.1 % stachyose while Sugimoto (1970) obtained 1.0 % raffinose and 3.2 % stachyose.

This study shows that the amount of stachyose hydrolysed is more than the amount of raffinose hydrolysed at the same operational conditions. This is an

advantage as the stachyose content in soymilk is greater than raffinose. Previous studies observed that stachyose induces more flatulence than raffinose.

Consumption of soy products containing 1.32 g to 3.06 g of raffinose and stachyose did not considerably increase flatulence frequency. Saurez, *et al* (1995), reported that the consumption of 100 ml of unprocessed soymilk causes flatulence, but the processed soymilk may not. 1-2 % reduction in raffinose and stachyose was adequate to overcome flatulence. Thus flatulence was not caused by soymilk processed by peanut α- galactosidase.

6. FUTURE WORK

The enzyme used in this study was not purified and this is one of the reasons for lower hydrolytic activity of the immobilised enzyme. A further study could be done with purified enzyme and by optimising the reaction conditions. In this study, a single flow-rate was used for continuous hydrolysis. But it would be beneficial to do the same study at various flow rates and thereby comparing the hydrolysis rates. The efficiency of immobilised enzyme could be increased with the use of a cross-linking agent, which may reduce the leakage of enzyme from the beads.

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APPENDIX

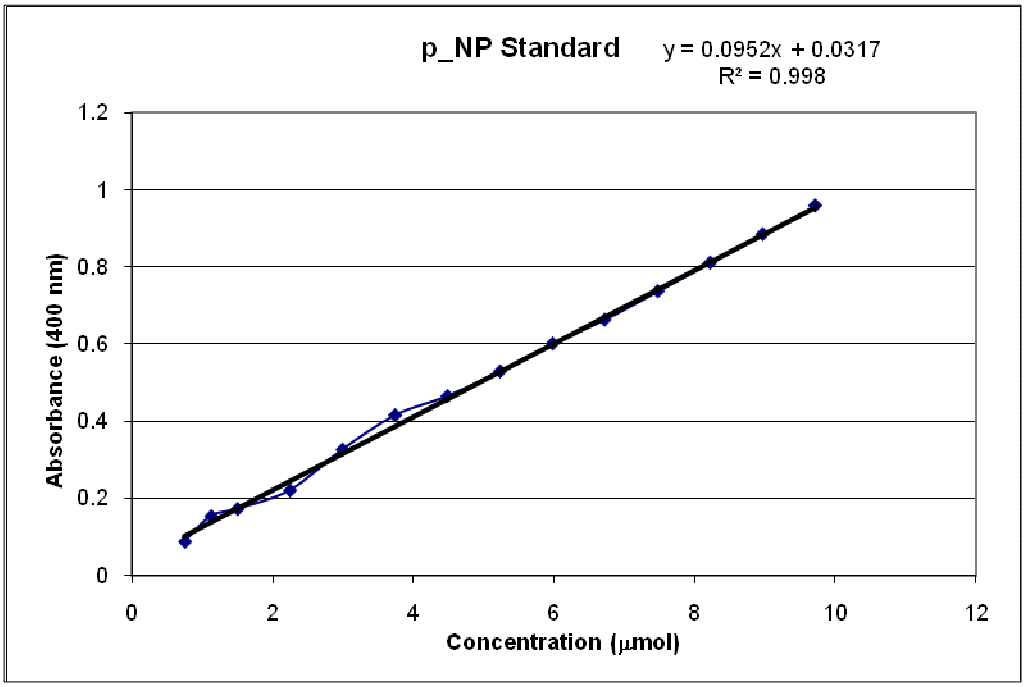

Figure A.1 p-NP standard graph

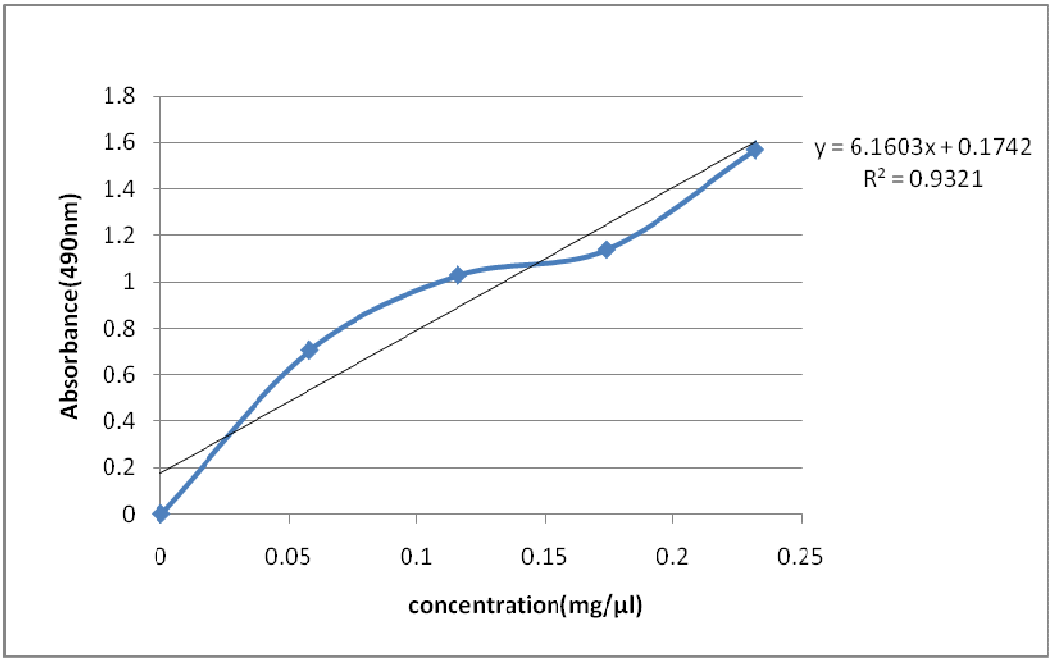

Figure A.2 Standard curve for stachyose

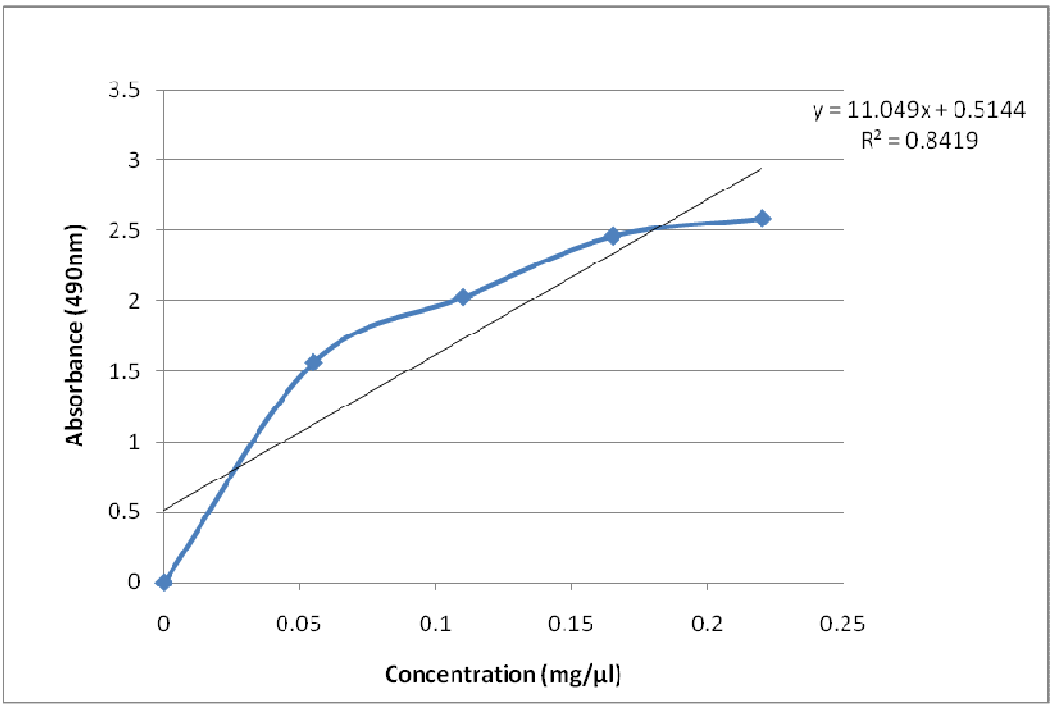

Figure A.3 Standard curve for raffinose